[CONTRIBUTION FROM LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

The Hydrolytic Action of Acylase I on N-Acylamino Acids[1]

BY SHOU-CHENG J. FU[2] AND SANFORD M. BIRNBAUM

RECEIVED JULY 7, 1952

Data concerning the relationship of the structure of simple N-acylated aliphatic amino acids and their susceptibility to hydrolysis by hog kidney acylase I is summarized. A plot of the logarithm of the rate of hydrolysis of N-chloroacetylamino acids *versus* the calculated inductive constant reveals a linear relationship for the straight chain amino acids up to 5 carbon atoms. When the N-chloroacetylamino acid possesses a longer or more branched chain, the gradually increasing bulkiness of the molecule progressively hinders the hydrolysis. The effect of different substituents on the amino acid moiety is also demonstrated. A hypothesis is proposed concerning the spatial configuration of the N-acylamino acid molecule under the influence of the enzyme prior to the actual hydrolysis.

Studies on the relative susceptibility of a wide variety of N-acylated amino acids to the hydrolytic action of hog kidney acylase I[3] have revealed the following: (a) with the same acyl radical the hydrolysis rates vary widely with the nature of the amino acid; (b) with the same amino acid the rates vary with the nature of the N-acyl radical, the highest rates so far observed being with the N-chloroacetyl derivatives; (c) in the straight chain aliphatic, homologous series, *i.e.*, glycine, alanine, α-aminobutyric, etc., the N-acylated derivatives are split at regularly increasing rates to a maximum at *n*-valine, decreasing with the larger chain derivatives[4]; (d) branching of the amino acid side chain invariably depresses the rate below that of the corresponding straight chain isomer; (e) the N-acyl-D-amino acids are either completely resistant or are attacked at negligibly slow rates; and (f) the allostereoisomers of threonine and isoleucine are hydrolyzed at different rates from the corresponding normal forms.

TABLE I

Compounds	Initial rates of hydrolysis, μmoles/hr./mg. N
Group I[a]	
N-Chloroacetylisopropylamine	0
N-Chloroacetyl-DL-alanine amide	0
N-Chloroacetyl-DL-alanine ethyl ester	4
Group II	
N-Chloroacetyl-α-aminoisobutyric acid	11
N-Chloroacetyl-DL-α-amino-α-methyl-*n*-butyric acid	37
N-Chloroacetyl-DL-α-amino-α-methyl-*n*-valeric acid	31
N-Chloroacetyl-DL-α-amino-α-methyl-isovaleric acid	3.5
N-Chloroacetyl-α-amino-α-ethyl-*n*-butyric acid	0
Group III	
N-Chloroacetyl-N-methyl-DL-alanine	0
N-Chloroacetyl-N-ethyl-DL-alanine	0
Group IV	
N-Chloroacetyl-DL-*tert*-leucine	300

[a] Other compounds in this category studied were N-chloroacetyl-*n*-propylamine, N-acetyl-*t*-hexylamine, N-chloroacetyl-*t*-hexylamine and N-chloroacetylleucine amide. Their susceptibility to acylase I was zero.

With the aim of extending this study and further elucidating the relation of substrate structure to enzyme susceptibility we have prepared a number of new N-acylated amino acids and their derivatives and have determined their hydrolytic rates as before.[3] From these and earlier findings, possible conclusions concerning the relative hydrolytic action of the enzyme have been considered.

Table I gives the initial rates of hydrolysis of N-chloroacetylamino acids and related compounds not previously published.

It is apparent from the figures given in group I that the free carboxyl is essential for the enzymatic action of acylase I. The chloroacetylamines may be considered amino acid derivatives in which the carboxyl group is reduced to a methyl group. N-Chloroacetylalanine ethyl ester on incubation with acylase I yielded 4 μ moles of *free* amino acid per hour per mg. of protein N, indicating the presence of a weak esterase activity.

Group II consists of N-chloroacetylamino acids without an α-hydrogen atom. When the α-hydrogen is substituted by a methyl group, a slow but definite asymmetric hydrolysis is observed.[5,6] Substitution by an ethyl or larger group completely inhibits enzymatic hydrolysis.

Replacement of the peptide hydrogen by an alkyl group (group III) is more critical. No detectable splitting could be observed with the chloroacetyl derivatives of either N-methyl- or N-ethylalanine. This finding is consistent with the very low rate earlier observed with chloroacetyl-L-proline.[3]

The Inductive Effect.—The inductive constant, σ, of the amino nitrogen of those amino acids with simple hydrocarbon side chains can be calculated by the equation

$$\sigma = \Sigma I_C \alpha^i + I_{C\langle O \atop OH} \alpha^i$$

where

$$I_C = -0.4,\ I_{C\langle O \atop OH} = 6.5,\ \alpha = 1/2.8^7$$

and i = number of atoms separating the inducing atom from the amino-nitrogen. When plotted against the inductive constant, the logs of the rates

(1) Presented in part before the Biological Chemistry Division of the American Chemical Society, 121st Meeting, Milwaukee, March, 1952.

(2) Visiting Scientist, National Cancer Institute.

(3) S. M. Birnbaum, L. Levintow, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952).

(4) C. G. Baker and A. Meister, THIS JOURNAL, **73**, 1336 (1951).

(5) N-Chloroacetyl-DL-isovaline has been resolved in this Laboratory by the use of acylase I (*cf.* 3) and the L- and D-isomers of isovaline prepared in pure form.

(6) C. G. Baker, S-C. J. Fu, S. M. Birnbaum, H. Sober and J. P. Greenstein, THIS JOURNAL, **74**, 4701 (1952).

(7) G. E. K. Branch and M. Calvin, "The Theory of Organic Chemistry," Prentice-Hall, Inc., New York, N. Y., 1947, p. 204.

of hydrolysis of the chloroacetyl derivatives of these amino acids fall along two distinct straight lines. The exceptions noted are those amino acids without α-hydrogen.

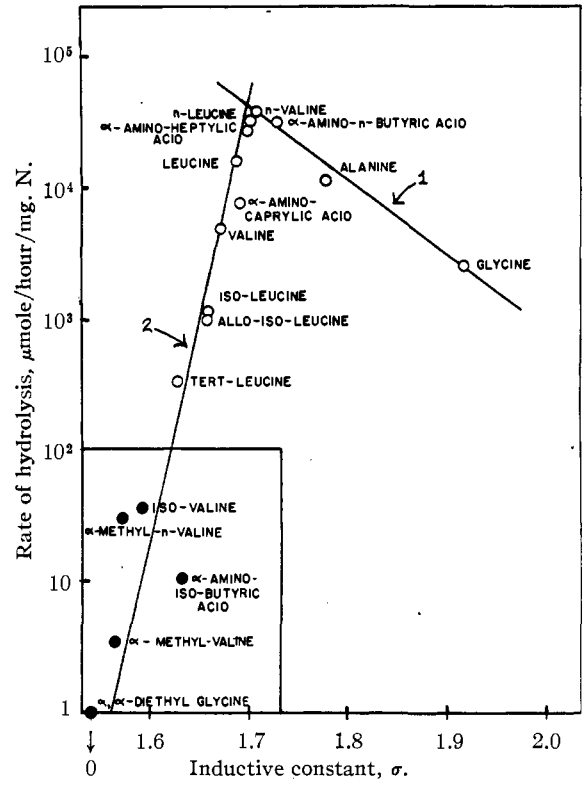

Fig. 1.—Rate of enzymic hydrolysis of N chloroacetyl-DL-amino acids by acylase I *vs.* inductive constant of amino-nitrogen.

If we can assume that the ease of hydrolysis of the peptide bond is proportional to increasing electronegativity of the amino-nitrogen (line 1) then line 2 must be the resultant of regularly increasing steric hindrance interfering at some other point in the enzyme substrate interaction and overcoming the theoretically increasing susceptibility of the peptide bond to hydrolysis. This would then be a rather precise measure of the steric hindrance for this series of compounds.[8]

Bergmann[9,10] has proposed a planar arrangement of the dipeptide molecule to account for the optical specificity and other phenomena of dipeptidases. A modification and extension of this hypothesis serves equally well to explain some of the above observations concerning the enzymic action of acylase I. Figure 2 describes the planar substrate configurations assumed for this purpose. To ascribe an effect of the side chain on the rate of hydrolysis, other than that of its inductive effect, it might be assumed that two pincer-like limbs (**a** and **b**) need a perfect fit around the asymmetric carbon, where both the α-hydrogen and R are involved. The substitution of a methyl or larger group for the α-hydrogen which prevents the enzyme from having proper contact on the polar (upper) side of the molecule by limb **a** results in a strong diminution of the enzymic hydrolysis. The gradually increasing bulkiness of R below the plane, contacted by limb **b**, makes the fitting increasingly difficult. Consequently the rate of hydrolysis decreases linearly with the increasing size of the branched chain. This is observed where the chain length increases beyond 5 carbon atoms or the branching point of the side chain approaches toward the α-carbon.

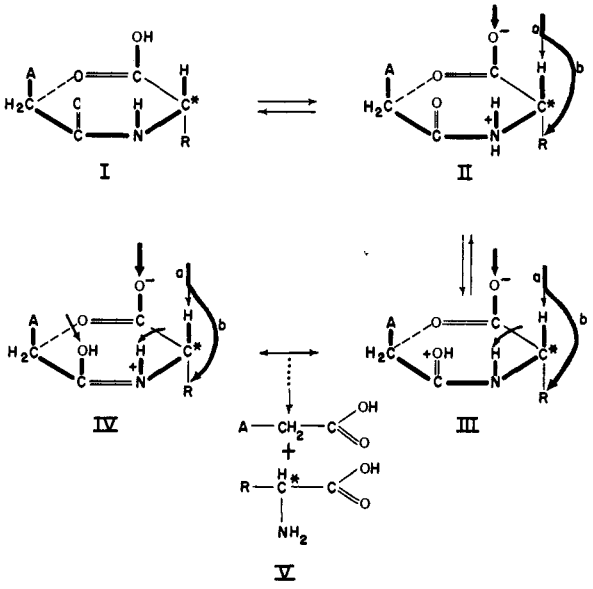

Fig. 2.—Planar configurations of acylase I substrate: A = Cl or H; R = aliphatic substituent; ------- indicates only the spatial arrangement of the molecule into a hexagon.

The ionization of the carboxyl group transforms the substrate molecule from I to the dipolar ionic structure II. The ionized form is in equilibrium with III which is a resonating hybrid of form IV. The probable formation of a substrate–enzyme complex is more favorably accomplished at III because of the higher electronegativity of the amino-nitrogen. The final cleavage, from III and IV, is probably analogous to that of the hydrolysis of amides,[11] leading to the end-products V.

It would be expected that the polarization of the carbonyl group and the formation of the imido structure IV would be facilitated by the presence of a strong electron-attracting terminal, A, on the acyl end of the substrate molecule. This is in agreement with our observation that, invariably, the chloroacetylamino acids are hydrolyzed at a greater rate than the acetyls.

Experimental

Preparation of Acylated Amino Acids. (1).—α-Amino-α-methyl-*n*-butyric acid (isovaline), α-amino-α-methyl-*n*-valeric acid (α-methyl-*n*-valine), α-amino-α-methyl-isovaleric acid (α-methylvaline) and α-amino-α-ethyl-*n*-butyric acid (α,α-diethylglycine) were prepared according to the modified Zelinsky and Stadnikoff method[12] reported

(8) Although this interpretation is based solely on steric effect, the possibility of a change in reaction mechanism at the point of inversion of the curve cannot be ruled out at present. Work on this phase is being continued.

(9) M. Bergmann, "The Harvey Lectures," 1935–1936, p. 37.

(10) M. Bergmann, L. Zervas, J. S. Fruton, F. Schneider and H. Schleich, *J. Biol. Chem.*, **109**, 325 (1935).

(11) C. R. Noller, "Chemistry of Organic Compounds," W. B. Saunders Co., 1951, p. 241.

(12) N. Zelinsky and G. Stadnikoff, *Ber.*, **39**, 1922 (1906).

by Levene and Steiger.[13] Equimolar proportions of the corresponding ketone, ammonium chloride and potassium cyanide were allowed to react in an excess of saturated ammonium hydroxide solution in a sealed flask for 6 hours at 60°. The amino acids were obtained in the form of their hydrochlorides which subsequently were treated with a slight excess of silver carbonate and filtered. The excess silver was then removed as silver sulfide. The purified amino acids showed properties identical with those described in the literature.[13,14]

(2) ***t*-Leucine (β,β,β-Trimethylalanine).**—Knoop's procedure[15] of aminating β,β,β-trimethylpyruvic acid in 25% alcohol solution saturated with ammonia in the presence of Pd catalyst, failed to yield the reported compound. However, Knoop's earlier synthesis[16] of this acid by reduction of oxime of β,β,β-trimethylpyruvic acid with aluminum amalgam yielded a product in 70% yield. The extremely low yield of the oxime makes this synthesis undesirable.

(3) ***t*-Hexylamine (2-Amino-3,3-dimethylbutane) Hydrochloride.**—An almost quantitative yield of *t*-hexylamine hydrochloride was obtained by reduction of pinacolone oxime[17] in sodium–absolute ethanol.[18] The crude hexylamine hydrochloride was recrystallized from water and alcohol and used for later acylation.

(4) **N-Methyl- and N-Ethylalanine.**—One mole of α-bromopropionic acid was treated with 6 times its volume of the corresponding 30% amine solution for 48 hr. at 40°. The crude N-alkylalanine was obtained by evaporating off the excess amine and water under reduced pressure. The final product was recrystallized from water.

(5) **Acetylation and Chloroacetylation.**—The preparation of N-acetyl- and N-chloroacetylamino acids were carried out in the usual manner.[19] The N-chloroacetylamino acid ester and amides were also prepared by the interaction of chloroacetyl chloride and the ester or amide in absolute alcohol solution with strong agitation.

The chloroacetylamines were prepared by addition of chloroacetyl chloride, in portions into an equal molar solution of free amine dissolved in dry ether, at 0° with stirring. The reaction mixture was then gently heated on a steam-bath for 20 min. to complete the reaction. After the removal of the solvent, the chloroacetylated amine was purified by distillation under diminished pressure.

In case an amine hydrochloride was used, the equivalent amount of hydrochloride was neutralized with strong sodium hydroxide solution and extracted with ether. The ether extract was dried and filtered before use.

N-Chloroacetyl-α-amino-isobutyric acid, m.p. 149°. *Anal.*[20] Calcd. for $C_6H_{10}O_3NCl$: C, 40.16; H, 5.61; N, 7.80; Cl, 19.74. Found: C, 40.22; H, 5.59; N, 7.72; Cl, 19.63.

N-Chloroacetyl-β,β,β-trimethylalanine, m.p. 204°. *Anal.* Calcd. for $C_8H_{14}O_3NCl$: C, 46.29, H, 6.80; N, 6.76; Cl, 17.06. Found: C, 46.20; H, 6.70; N, 6.69; Cl, 17.05.

N-Chloroacetyl-α-amino-α-methyl-*n*-butyric acid,[21] m.p. 162°. *Anal.* Calcd. for $C_7H_{12}O_3NCl$: C, 43.42; H, 6.25; N, 7.24; Cl, 18.31. Found: C, 43.41; H, 6.42; N, 7.20; Cl, 18.18.

N-Chloroacetyl-α-amino-α-methyl-*n*-valeric acid, m.p. 165–166°. *Anal.* Calcd. for $C_8H_{14}O_3NCl$: C, 46.29; H, 6.80; N, 6.76; Cl, 17.06. Found: C, 46.24; H, 6.79; N, 6.72; Cl, 17.10.

N-Chloroacetyl-α-amino-α-methyl-isovaleric acid, m.p. 167°. *Anal.* Calcd. for $C_8H_{14}O_3NCl$: C, 46.29; H, 6.80; N, 6.76; Cl, 17.06. Found: C, 46.11; H, 6.73; N, 6.90; Cl, 17.05.

N-Chloroacetyl-α-amino-α-ethyl-*n*-butyric acid,[19] m.p. 190°. *Anal.* Calcd. for $C_8H_{14}O_3NCl$: C, 46.29; H, 6.80; N, 6.76; Cl, 17.06. Found: C, 46.19; H, 6.86; N, 6.83; Cl, 17.17.

N-Chloroacetyl-*n*-propylamine, b.p. 90–91° (4 mm.). *Anal.* Calcd. for $C_5H_{10}ONCl$: C, 44.28; H, 7.43; N, 10.33; Cl, 26.15. Found: C, 44.20; H, 7.40; N, 10.25; Cl, 26.30.

N-Chloroacetylisopropylamine, m.p. 42°. *Anal.* Calcd. for $C_5H_{10}ONCl$: C, 44.28; H, 7.43; N, 10.33; Cl, 26.15. Found: C, 44.30; H, 7.40; N, 10.12; Cl, 26.25.

N-Acetyl-*t*-hexylamine, m.p. 70–71°. *Anal.* Calcd. for $C_8H_{17}ON$: C, 67.07; H, 11.98; N, 9.78. Found: C, 67.27; H, 12.12; N, 9.73.

N-Chloroacetyl-*t*-hexylamine, m.p. 79°. *Anal.* Calcd. for $C_8H_{16}ONCl$: C, 54.06; H, 9.08; N, 7.88; Cl, 19.95 Found: C, 53.96; H, 9.24; N, 7.95; Cl, 20.15.

N-Chloroacetyl-alanine amide, m.p. 146–147°. *Anal.* Calcd. for $C_5H_9O_2N_2Cl$: C, 36.48; H, 5.51; N, 17.02; Cl, 21.54. Found: C, 36.45; H, 5.60; N, 17.10; Cl, 21.62.

N-Chloroacetyl-N-ethylalanine, m.p. 143°. *Anal.* Calcd. for $C_7H_{12}O_3NCl$: C, 43.41; H, 6.27; N, 7.23; Cl, 18.31. Found: C, 43.20; H, 6.30; N, 7.32; Cl, 18.40.

Enzymic Studies.—The rates of enzymic hydrolysis of the new compounds reported here were determined in precisely the same manner as those taken from earlier work.[3] Three to five separate determinations were performed on each of the substrates involved using varying enzyme concentrations and incubation times. Rates calculated from the values thus obtained agreed to within 5% of the mean.

The digests were composed of 1 cc. of enzyme solution, 1 cc. of phosphate buffer at *p*H 7 and 1 cc. of neutralized 0.05 *M* racemic substrate. The time of incubation at 38° and the enzyme concentrations were so adjusted as to give no more than 30% hydrolysis of the available substrate. The hydrolysis of chloroacetyl-DL-alanine by this enzyme under these conditions follows zero order kinetics. Although complete kinetic measurements have not been performed for all of the N-acylated amino acids studied in this Laboratory (some 60 in all) the consistency shown by the increase in initial activity with acylase I over the activity of the original homogenate[22] leads to the assumption that the kinetics for all these N-acylated amino acids follow the same order.

No appreciable inhibition of the hydrolytic reaction by the end products was encountered. The fact that the reaction reached 100% hydrolysis of the L-forms of the N-acylated racemic amino acids was demonstrated for some 30 substrates by the evidence that the isolated L- and D-enantiomorphs were more than 99.9% optically pure.[3,23,24]

The actual amount of hydrolysis of the acyl amino acid was determined by the ninhydrin CO_2 method[25] with the following exceptions: (1) The splitting of acyl amino acids without α-hydrogen and acylamino acid amides and esters were determined by terminating the enzymic activity with an equal volume of 5% trichloroacetic acid, centrifuging off the precipitated protein, and determining the amino nitrogen formed in an aliquot of the supernate by the Van Slyke nitrous acid procedure.[26]

(2) It was found that N-alkyl alanine derivatives fail to give the theoretical amount of CO_2 when treated with ninhydrin under the conditions described.[22] Actually N-methylalanine gives about 30% and N-ethylalanine gives 9%. Any splitting of the chloroacetyl derivatives of these compounds could be detected, however, by the ninhydrin CO_2 methods using the appropriate correction factor.

BETHESDA 14, MD.

(13) P. A. Levene and R. E. Steiger, *J. Biol. Chem.*, **76**, 299 (1928).
(14) K. Kurono, *Biochem. Z.*, **134**, 434 (1922).
(15) F. Knoop and G. Landmann, *Z. physiol. Chem.*, **148**, 307 (1925).
(16) F. Knoop and G. Landmann, *ibid.*, **89**, 157 (1914).
(17) L. F. Fieser, "Experiments in Organic Chemistry," D. C. Heath and Co., New York, N. Y., 1941, p. 102.
(18) W. Ssolonina, *J. russ. phys.-chem. soc.*, **31**, 541 (1899).
(19) E. Fischer, *Ann.*, **340**, 123 (1905).
(20) The analyses were performed by Mr. James F. Williams and Miss Rita A. McCallum of this Laboratory.
(21) K. W. Rosenmund, *Ber.*, **42**, 4473 (1909).
(22) K. R. Rao, S. M. Birnbaum, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **198**, 507 (1952).
(23) A. Meister, L. Levintow, R. B. Kingsley and J. P. Greenstein, *ibid.*, **192**, 535 (1951).
(24) S. M. Birnbaum and J. P. Greenstein, *Arch. Biochem. Biophys.*, **39**, 108 (1952).
(25) D. D. Van Slyke, R. T. Dillon, D. A. MacFadyen and P. Hamilton, *J. Biol. Chem.*, **141**, 627 (1941).
(26) D. D. Van Slyke, *ibid.*, **83**, 425 (1929).